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SUBSCRIPTIONS

Microparticles harboring Sonic Hedgehog promote angiogenesis through the upregulation of adhesion proteins and proangiogenic factors

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Microparticles (MPs) are small fragments generated from the plasma membrane after cell stimulation or apoptosis. We have recently shown that MPs harboring the morphogen Sonic Hedgehog (MPs[Shh+]) correct endothelial injury by release of nitric oxide from endothelial cells [Agouni, Mostefai, Porro, Carusio, Favre, Richard, Henrion, Martínez and Andriantsitohaina (2007) FASEB J., 21, 2735–2741]. Here, we show that MPs[Shh+] induce the formation of capillary-like structures in an *in vitro* model using human endothelial cells, although they inhibited cell migration. Besides, MPs[Shh+] regulate cell proliferation. Both cell adhesion and expression of proteins involved in this process such as Rho A and phosphorylation of focal-activated kinase were increased by MPs[Shh+], via a Rho-associated coiled-coil-containing protein kinase inhibitor-sensitive pathway. We demonstrate that MPs[Shh+] increase messenger RNA and protein levels of proangiogenic factors as measured by quantitative reverse transcription–polymerase chain reaction and western blot. In spite of vascular endothelial growth factor expression, conditioned media from endothelial cells treated avec MPs[Shh+] reduces angiogenesis. Interestingly, the effects induced by MPs[Shh+] on the formation of capillary-like structures, expression of adhesion molecules and proangiogenic factors were reversed after silencing of the Shh receptor, using small interfering RNA or when Sonic Hedgehog (Shh) signaling was pharmacologically inhibited with cyclopamine. Taken together, we show that Shh carried by MPs[Shh+] regulate angiogenesis probably through both a direct and an indirect mechanisms, and we propose that MPs harboring Shh may contribute to the generation of a vascular network in pathologies associated with tumor growth.

Introduction

The morphogen Sonic Hedgehog (Shh) pathway is critical for normal growth. In addition to its role in embryonic development, it has been shown that, in adults, Shh network can participate in cell differentiation, proliferation and angiogenesis (1). Shh signal transduction is complex and not completely elucidated. In addition to the receptor, Patched-1, which represses the membrane protein Smoothened (Smo), other partners have been described such as Hedgehog-interacting protein (Hip) or Cam-related/downregulated by onco-genes. While Hip is able to bind Shh and to block Shh signaling (2), Cam-related/downregulated by oncogene increases Shh signaling (3). Regarding angiogenesis, it has been reported that Shh pathway activation promotes *in vitro* capillary-like structures on Matrigel® (4) but also *in vivo* neo-vascularization (5). Moreover, recent data showed that the effects of Shh recombinant protein through Smo receptor in both angiogenesis and specification of neuronal fates are mediated by the phosphatidylinositol 3-kinase/Akt but not protein kinase C pathways (4,6,7). Also, Hip is downregulated on human endothelial cells during capillary-like structure formation suggesting that regulation of level expression of Hip may play a role in angiogenesis (2).

Taking in consideration that angiogenesis is an essential process of cells from cardiovascular system during diseases associated with ischemia or for tumor growth, the role of Shh-induced angiogenesis in these pathologies is critical. Thus, it has been shown that Shh gene therapy may have considerable therapeutic potential by improving cardiac function in either ischemia or infarct models and wound healing in diabetes (8,9). In parallel to the effects of Shh cascade in angiogenesis, uncontrolled activation of the Hedgehog-signaling pathway is a causal factor in many cancers (10) and upregulation of Shh-related proteins has been shown in human tumors such as pancreatic adenocarcinoma, basal cell carcinoma, glioma and prostate cancer (11–14).

Tumor cells but also blood and vessel wall cells are able to release large amounts of small plasma membrane fragments called microparticles (MPs). MPs are responsible, at least in part, to cancer-associated thrombosis because they harbor at their surface tissue factor and other components necessary for thrombus formation (15). Thus, MPs may represent a sign of vascular complications in patients with lung and gastric cancer (16,17). These authors have reported enhanced circulating monocyte- and platelet-derived MPs in patients with lung cancer. In addition, levels of P-selectin associated to platelet MPs and tissue factor generated from cancer cells are increased indicating that proteins involved in hemostasis are elevated in patients with cancer (18) and may represent a tool for exacerbated thrombosis. Also, through proteins carried by MPs from tumor cells, such as urokinase, CD147 or sphingomyelin, MPs can modify the adhesive and invasive properties of target cells (19) or the angiogenic activity of endothelial cells (20). Moreover, it has been shown that platelet MPs enhance the *in vitro* invasive potential of breast cancer cell lines and induce metastasis and angiogenesis in lung cancer (21,22). These data suggest that MPs transfer a transcellular signal that may allow tumor progression. In this context, MPs from acute myelogenous leukemia patients functionally transferred C-X-C chemokine receptor to HL-60 cells and increased their chemotaxis and homing to the bone marrow of immunodeficient mice (23).

We have recently shown that MPs generated *in vitro* from human apoptotic/stimulated lymphocytes express Shh (MPs[Shh+]) at their surface and induce cell differentiation (24). In addition, this type of MPs is able to stimulate nitric oxide production from endothelial cells by direct activation of the Shh and phosphatidylinositol 3-kinase pathways (25). Thus, MPs[Shh+] could represent a potential tool to modulate angiogenesis through their direct action on endothelial cells. Here, we studied the effects of MPs[Shh+] on *in vitro* angiogenesis using a human model of endothelial cells. For this, we have investigated MPs[Shh+] effects on migration, proliferation, adhesion and formation of capillary-like structures as well as the angiogenic factors produced by endothelial cells under MPs[Shh+] treatment.

Materials and methods

MP production

The human lymphoid CEM T cell line (American Type Culture Collection, Manassas, VA) was used for MP production. Cells were seeded at 10^6 cells/ml and cultured in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD).

Abbreviations: FLT, fms-like tyrosine kinase; Hip, Hedgehog-interacting protein; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; MP, microparticle; mRNA, messenger RNA; PBS, phosphate-buffered saline; FAK, focal adhesion kinase; ROCK, Rho-associated coiled-coil-containing protein kinase; RT–PCR, reverse transcription–polymerase chain reaction; Shh, Sonic Hedgehog; siRNA, small interfering RNA; Smo, smoothened; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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MPs were produced as described previously (24,25). Briefly, CEM cells were treated with phytohemagglutinin (5 μg/ml; Sigma–Aldrich, St Louis, MO) for 72 h and then with phorbol-12-myristate-13 acetate (20 ng/ml; Sigma–Aldrich) and actinomycin D (0.5 μg/ml; Sigma–Aldrich) for 24 h. A supernatant was obtained by centrifugation at $750g$ for 15 min and then at $1500g$ for 5 min to remove cells and large debris, respectively. MPs from the supernatant were washed after three centrifugation steps (45 min at $14\,000g$) and recovered in 400 μl NaCl (0.9% wt/vol). Washing medium for the last supernatant was used as control. Determination of the amount of MPs was carried out by measuring MP-associated proteins, using the method of Lowry, with bovine serum albumin (Sigma–Aldrich) as the standard. Shh carried by MPs was detected by western blot as described previously (24) (data not shown).

Cell culture

The Eahy 926 endothelial cell line was maintained at 37°C in a humidified incubator gassed with 5% CO_2 in air and was cultured in growth medium (Dulbecco's modified Eagle's medium:Ham's F-12, 1:1; Lonza) supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% Na-pyruvate, 1% streptomycin/penicillin (Lonza), 1% hypoxanthine, aminopterin, thymidine (Sigma–Aldrich) and 10% of heat-inactivated fetal bovine serum (Invitrogen, Cergy Pontoise, France). Also, freshly delivered umbilical cords were obtained from a nearby hospital. Human umbilical vein endothelial cells (HUVECs) were obtained as described previously (26) and grown on plastic flasks in MCBD 131 medium containing 1% L-glutamine, 1% streptomycin/penicillin, 500 ng/l epidermal growth factor, 1 μg/l basic fibroblast growth factor, supplemented with 10% of heat-inactivated fetal bovine serum. HUVECs were used at the second to fourth passage. Cells were grown for 24 h in the absence or presence of 10 μg/ml MPs preincubated or not for 30 min with Smo inhibitor cyclopamine (30 μM; Biomol International, Plymouth Meeting, PA) or Rho-associated coiled-coil-containing protein kinase (ROCK) inhibitor (Y-27632, 10 μM). All agents were used at concentrations at which no cytotoxicity was observed, as deduced from Trypan blue exclusion and with 3-(4,5-dimethylth-iazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In our previous study, treatment of endothelial cells *in vitro* with 10 μg/ml MPs for 24 h corresponded to the concentration and time required to obtain nitric oxide release from endothelial cells (25). Thus, all the experiments of the present work were performed under these conditions.

In vitro capillary network formation on Matrigel®

After 24 h of incubation with MPs in the absence or in the presence of the inhibitor cyclopamine, small interfering RNA (siRNA) (see below), basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, 10 ng/ml) or vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA, 20 ng/ml), Eahy 926 cells and HUVECs were detached with trypsin ethyl-enediaminetetraacetic acid (Lonza). Cells were seeded with a density of 150×10^3 cells per well precoated with Matrigel® (Sigma–Aldrich). Briefly, 100 μl of Matrigel® substrate diluted with serum-free medium (1:1 dilution) was added into a four-well plate and allowed to solidify for 1 h at 37°C. Then, cells were incubated with medium containing 10% of fetal bovine serum and allowed to adhere for 1 h after which the different stimuli were added. Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 4

and 24 h and quantified using ImageJ software. To determine whether mediators secreted by cells treated with MPs are able to induce capillary formation, HU-VECs were treated with MPs or cyclopamine + MPs as described above and culture media were removed. Then, HUVECs were treated with the conditioned medium for 24 h, and tube formation was determined as described above.

RNA interference and transient transfection

In order to silencing Patched-1, the Shh receptor, siRNA duplexes specific for human Patched-1 and control, non-silencing siRNA was obtained from Santa Cruz Biotechnology. Transient transfection of Eahy 926 endothelial cells and HUVECs was done according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates, grown for 24 h (60% confluence) and then transiently transfected with 100 nM of Patched-1-specific or control siRNA using the trans-fection reagent provided, which also served as control without siRNA. Medium was replaced 24 h later by fresh medium and cells were grown for an additional 24 h, prior to either western blot analysis of Patched-1 expression or functional studies. After siRNA transfection, Patched-1 downexpression was >80% (data not shown) as previously illustrated by Agouni *et al.* (25).

Western blot

After treatment, cells were homogenized and lysed. Proteins (20 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Blots were probed with anti-Rho A (Santa Cruz Biotechnology), anti-p-focal adhesion kinase (Y925) (p-FAK, Cell Signaling, Danvers, MA), anti-Patched-1, intercellular adhesion molecule (ICAM)-1, VEGF, fms-like tyrosine kinase (FLT)-1 and interleukin (IL)-1β (Santa Cruz Biotechnology) antibodies. Monoclonal anti-β-actin antibody (Sigma–Aldrich) was used at 1:2000 dilution to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein–antibody complexes were detected by Enhanced chemiluminescence plus (Amersham Biosciences) according to the protocol of the manufacturer.

Migration assay

Eahy 926 cells were seeded and grown to confluence in cell culture medium. After 24 h of serum starvation, cells were rinsed three times with serum-free

Table I. Genes analyzed by RT–PCR (alphabetical order)

α5 integrin	FGF1	FLT-1	MCP-1	SDF1
ANG1	FGF2	FLT-4	MMP1	TGFβ1
ANG2	FGF3	HGF	MMP2	TGFβ2
angiogenin	FGF5	ICAM-1	PDGFA	Tie1
β3 integrin	FGF7	IGF-1	PDGFB	Tie2
CCR2	FGF8	IL-1β	PDGFRb	TSP1
edg1	FGF10	IL-6	PECAM-1	VCAM-1
ephrin b2	FGFR1	IL-8RB	PEDF	VE-cadherin
E-selectin	FGFR2	KDR	RANTES	VEGF (A–D)
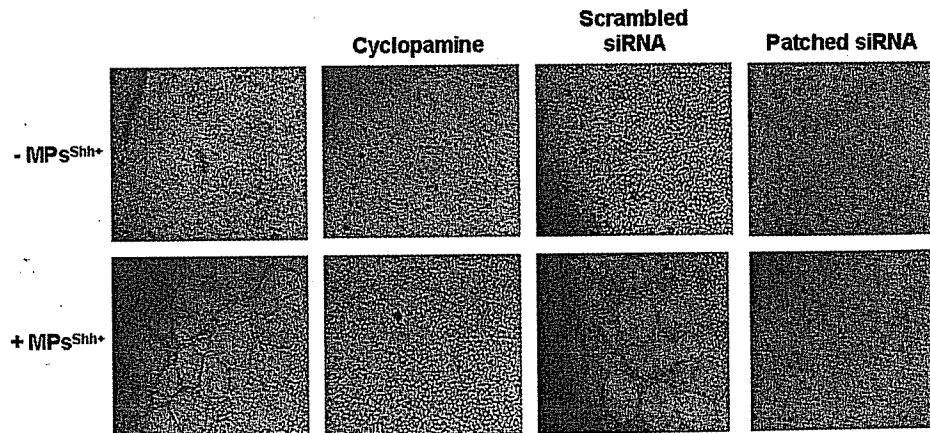
Fig. 1. MPs promote *in vitro* tube formation of Eahy 926 endothelial cells. Phase-contrast micrographs showing that 10 μg/ml MPs carrying Shh induce network formation on Matrigel® in Eahy 926 cells. Silencing Shh pathway by either the pharmacological inhibitor cyclopamine (30 μM) or siRNA of the Shh receptor, Patched-1, prevented MP-induced capillary formation. However, siRNA scrambled had no effect on *in vitro* angiogenesis evoked by MPs. Reproducible data were obtained from four independent experiments.

A

B
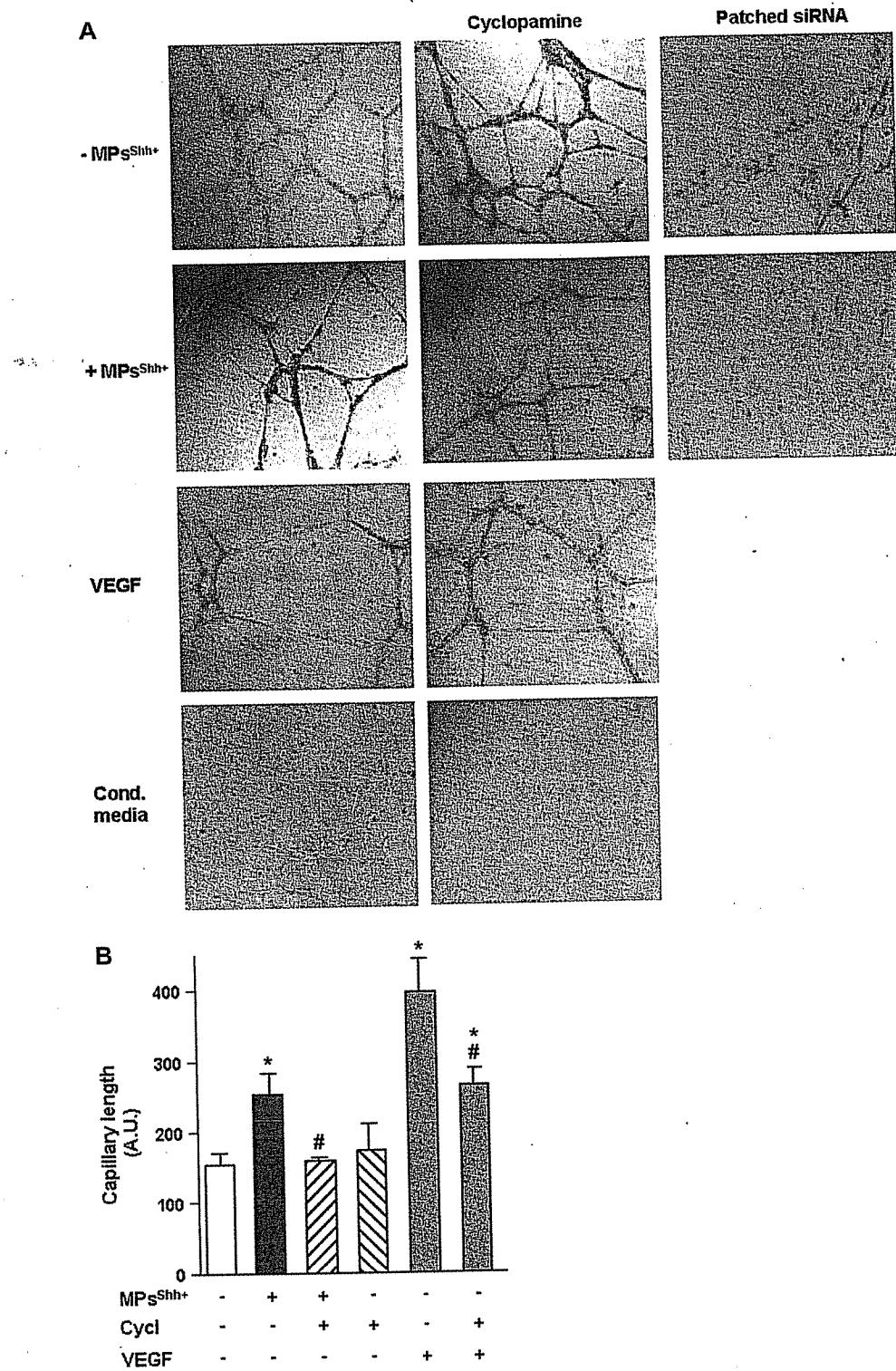
Fig. 2. MPs enhance *in vitro* tube formation of HUVECs. (A) Phase-contrast micrographs showing that 10 µg/ml MPs carrying Shh favor network formation on Matrigel® in HUVECs. Silencing Shh pathway with the pharmacological inhibitor cyclopamine (Cycl, 30 µM) or siRNA of the Shh receptor, Patched-1, prevented MP-induced capillary formation. Conditioned media obtained from HUVECs treated with MPs were unable to induce capillary formation. VEGF (20 ng/ml) was used as positive control. (B) Capillary length was used to quantify angiogenesis. Reproducible data were obtained from three independent experiments. $^*P < 0.05$ versus in the absence of MPs^{Shh+}. $^{\#}P < 0.05$ versus in the presence of MPs^{Shh+} alone or VEGF alone.

medium and then incubated with supplemented medium. The monolayer cell was wounded with a sterile pipet tip to make a gap as described previously (27). Then, detached cells were removed by washing, and cells were cultured in the absence or in the presence of MPs, MPs with cyclopamine or VEGF (20 ng/ml) as positive control. After 48 h, three selected non-adjacent fields at the lesion border were acquired using a ×10 phase objective on an inverted microscope (MOTIC AE21).

Confocal microscopy
After treatment, cultured cells were fixed with CELLFIX 1× (BD Biosciences, Le Pont de Claix, France) solution for 15 min at room temperature in culture dishes, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) and then blocked with 5% non-fat milk in PBS for 1 h at room temperature. The cells were treated with a rabbit polyclonal p-FAK (Y925) antibody

or mouse monoclonal anti-Rho A antibody in 5% non-fat milk in PBS for 1 h at room temperature. After washing with PBS, cells were treated with Alexa 488-conjugated goat anti-rabbit antibody (Interchim, Montluçon, France) and Alexa 488-conjugated goat anti-mouse (Interchim) in 5% non-fat milk in PBS for 1 h. In another set of experiment, tetramethylrhodamine isothiocyanate–phalloidin (Sigma–Aldrich) was used in order to label actin fibers. Briefly after treatments, cells were fixed with 2% paraformaldehyde and then stained with phalloidin (50 μg/ml) for 90 min at room temperature. After washing with PBS, the cells were mounted and visualized with a confocal microscopy (MRC-1024ES confocal equipment mounted on a Nikon Eclipse T3 300 microscope) in the Service Commun d'Imageries et d'Analyses Microscopiques from Angers University. All images were acquired using a ×60 objective.

Quantitative real-time reverse transcription–polymerase chain reaction analysis

Eahy 926 cells were grown for 24 h in the absence or presence of 10 μg/ml MPs preincubated or not with cyclopamine. Cells were detached using trypsin and after two subsequent steps of centrifugation at $500g$ for 10 min, the pellet containing cells were frozen in liquid N_2 and used to investigate the expression of messenger RNA (mRNA) for 46 transcripts related to angiogenesis by real-time reverse transcription–polymerase chain reaction (RT–PCR) (Table I). RT–PCR analyses were carried out by Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University, using a Chromo 4™ (Bio-Rad, Hercules, CA) and SYBR Green detection. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quanti-

fications were realized according to the ΔCt method and the relative gene expression levels were normalized using the geometric mean of three housekeeping genes as described previously (28).

Proliferation assay

Effects of MPs on proliferation on Eahy 926 cells or HUVECs were analyzed by using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, 10×10^3 cells per well were seeded into 96-well plates and allowed to attach overnight and then cells were treated with MPs for 24 h. After growth medium removal, dye-binding solution was added into each microplate well and cells were incubated at 37°C for 30 min. The fluorescence levels were read on a fluorescent microplate reader (Synergy HT, Biotek, Winooski, VT) with filters for ~485 nm excitation and ~530 nm emission.

Adhesion assay

Evaluation of adherent cells was performed using crystal violet staining (29). For adhesion experiments, 10×10^3 cells per well were seeded into 96-well plates for 24 h before addition of the stimuli, in the absence or in the presence of coated fibrinogen (Sigma–Aldrich, 100 μg/ml) (30). After 24 h of incubation, the plate was shacked for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% bovine serum albumin in medium without serum). Attached cells were fixed with 4% of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma–Aldrich) (1 mg/ml in 2% of ethanol) for 10 min at room temperature and extensively washed with
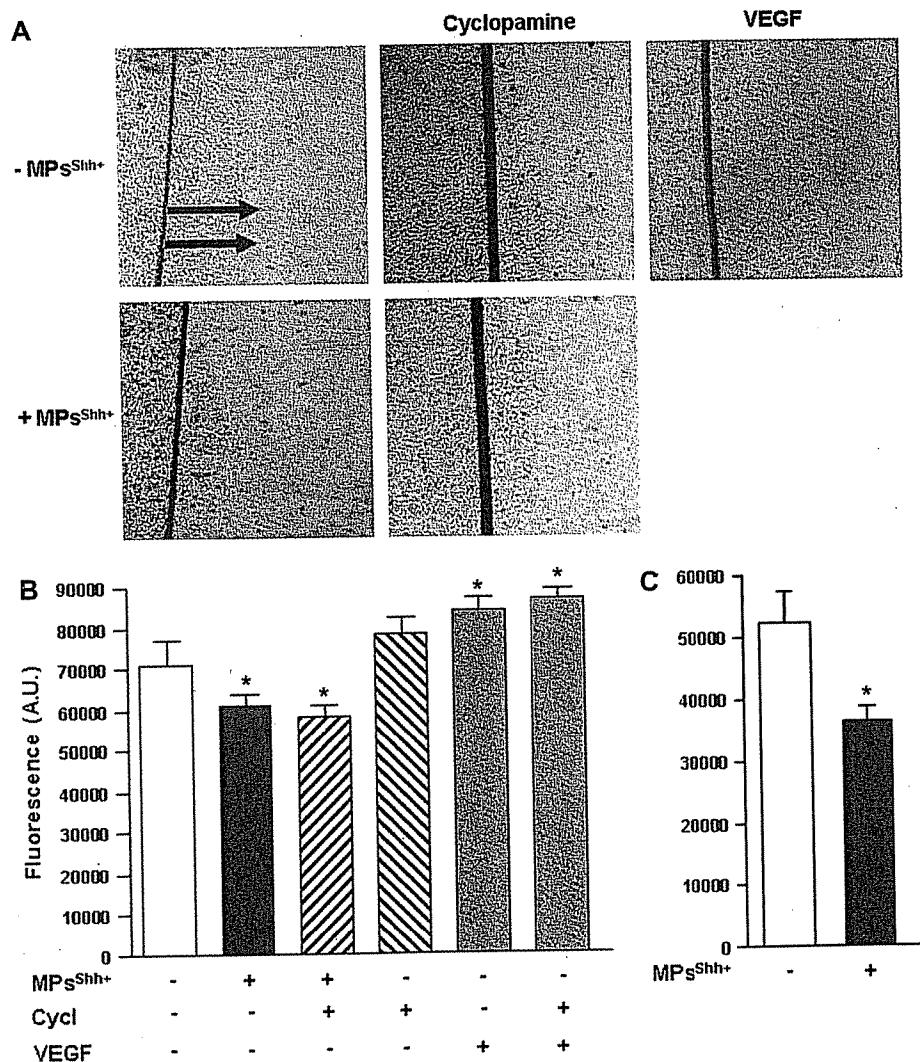
Fig. 3. MPs^Shh+ decrease cell migration and proliferation. (A) Ten micrograms per milliliter MPs^Shh+ treatment decrease cell migration when compared with the control conditions (in the absence of MPs^Shh+). The effects of MPs^Shh+ were not modified by cyclopamine (30 μM). VEGF (20 ng/ml) was used as positive control. Reproducible data were obtained from four independent experiments. (B) In Eahy cells, antiproliferative effects of MPs^Shh+ were not inhibited by treatment with cyclopamine (Cycl), whereas VEGF was able to increase cell proliferation by a mechanism insensitive to cyclopamine. (C) MPs^Shh+ treatment decreased proliferation on HUVECs. Results are means ± SEMs from six independent experiments. *$P < 0.05$ versus in the absence of MPs^Shh+.

distilled water. Then sodium dodecyl sulfate 2% was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Sinergy HT Biotek).

Statistical analysis

Data are represented as mean ± SEM, n represents the number of experiments repeated at least in triplicate. Statistical analyses were performed by Mann–Whitney U-tests (non-parametric). $P < 0.05$ was considered to be statistically significant.

Results

MPs^{Shh+} promote the formation of capillary-like structures

As shown in Figure 1, in the absence of treatment, endothelial Eahy cells failed to organize in capillary-like structures. After 24 h of treatment with MPs^{Shh+}, endothelial Eahy cells reorganized and formed capillaries on Matrigel®. Although in the absence of treatment HUVECs were able to form capillaries, MPs^{Shh+} enhanced capillary-like structure formation as illustrated by the capillary length measurement (Figure 2B). In order to determine whether Shh accounts for the effects evoked by MPs^{Shh+}, we examined the effect of Shh inhibition on MPs^{Shh+}-induced angiogenesis. Treatment of endothelial cells either with the selective Shh inhibitor, cyclopamine (30 μM), or siRNA to Shh receptor, Patched-1, resulted in an abolition of MPs^{Shh+} effects on capillary-like structure formation in both Eahy cells and HUVECs (Figures 1 and 2). It should be noted that cyclopamine alone or siRNA scrambled had no effect on endothelial cell angiogenesis. Furthermore, VEGF (20 ng/ml) promoted formation of capillaries on HUVECs and these effects were

partially inhibited by cyclopamine, suggesting a link between VEGF and Shh pathways (Figure 2). In addition, conditioned media from HUVECs treated with MPs^{Shh+} reduced the ability of these cells to form capillary-like structures (Figure 2A). Together, these findings suggest that the effects of MPs^{Shh+} are directly mediated by the Shh cascade.

MPs^{Shh+} reduce both endothelial cell migration and proliferation

Migration of endothelial cells, which allows cells to disseminate from the pre-existing vessel to form new vessels, contributes to angiogenesis. We studied the effects of MPs^{Shh+} on endothelial cell migration using a model of wound healing. MPs^{Shh+} slightly reduced migration of endothelial Eahy cells, which was not affected by cyclopamine (Figure 3A). Similar results were obtained in HUVECs treated with MPs^{Shh+} (data not shown). As positive control, VEGF was able to increase endothelial cell migration (Figure 3A).

Also, the effect of MPs^{Shh+} on cell proliferation was investigated since this process represents a critical step in angiogenesis. Figure 3B and C illustrates the antiproliferative effect of MPs^{Shh+}. MPs^{Shh+} were able to reduce cell proliferation by ~20 and 30% for Eahy cells (Figure 3B) and HUVECs (Figure 3C), respectively. In addition, cyclopamine treatment did not modify the inhibitory effect of MPs^{Shh+} on cell proliferation. These data indicate that Smo receptor is not implicated in the MPs^{Shh+} effects on endothelial cell proliferation. Furthermore, VEGF treatment induces an increase of cell proliferation, which was not affected by cyclopamine treatment (Figure 3B).
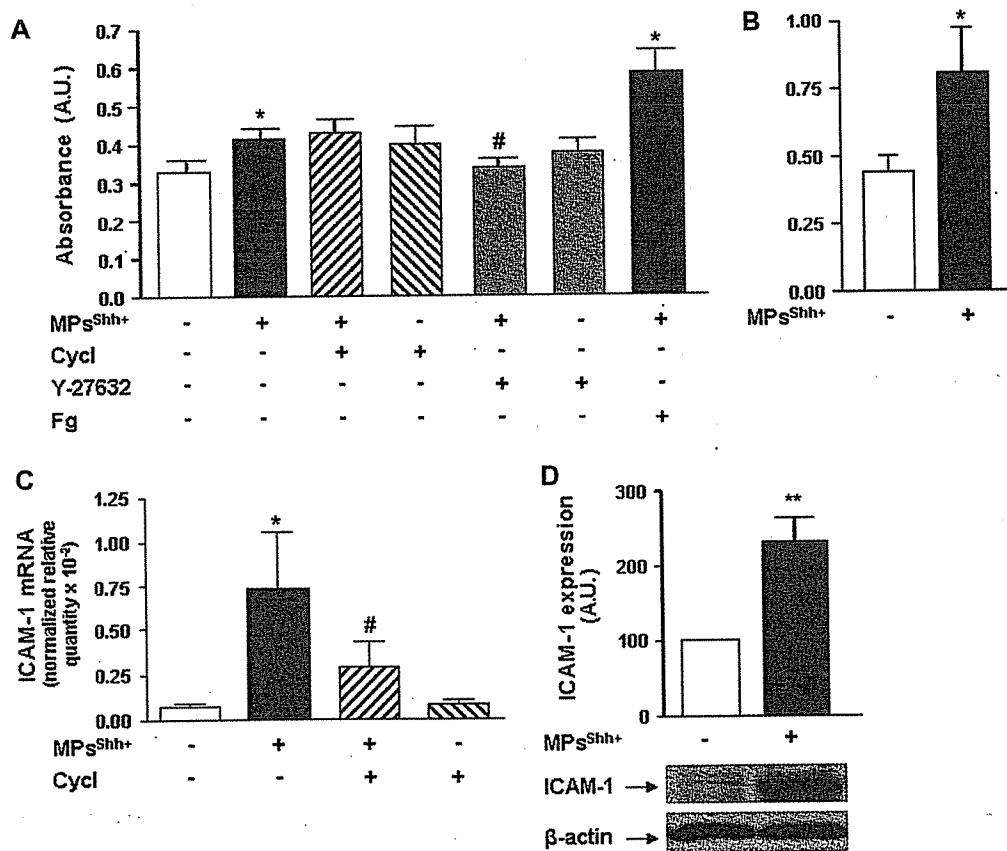
Fig. 4. MPs^{Shh+} increase adhesion of endothelial cells. Ten micrograms per milliliter MPs^{Shh+} enhance the number of Eahy cells (A) and HUVECs (B) that resulted positive to crystal violet staining. Treatment with the ROCK inhibitor (Y-27632, 10 μM) but not cyclopamine (Cycl, 30 μM) prevented the MPs^{Shh+}-induced increase of endothelial cell adhesion. In fibrinogen (Fg)-coated plates, adhesion was enhanced by MPs^{Shh+} treatment. Results are means ± SEMs from six independent experiments. *$P < 0.05$ versus in the absence of MPs^{Shh+}. #$P < 0.05$ versus in the presence of MPs^{Shh+} alone. (C) Quantitative RT–PCR analysis was conducted on total RNA from six independent Eahy cultures. ICAM-1 mRNA expression levels were enhanced by MPs^{Shh+} treatment. Cyclopamine partially reversed the MPs^{Shh+}-induced increase of ICAM-1 mRNA expression. *$P < 0.05$ versus in the absence of MPs^{Shh+}. #$P < 0.05$ versus in the presence of MPs^{Shh+} alone. (D) Western blot showing ICAM-1 protein level expression in the presence of MPs^{Shh+}. ICAM-1 protein expression was increased by MPs^{Shh+} treatment. **$P < 0.01$ versus in the absence of MPs^{Shh+}.

MPs^{Shh+} upregulate proteins involved in cell adhesion through Rho A and Shh pathways

Adhesion assay using crystal violet shows that MPs^{Shh+} increased adhesion in both Eahy and HUVECs cells (Figure 4A and B, respectively). Although cyclopamine was not able to reduce the effects of MPs^{Shh+} on cell adhesion, inhibition of ROCK by Y-27632 (10 µM) decreased MPs^{Shh+}-induced cell adhesion (Figure 4A). Specific transcripts for the ICAM-1 measured by real-time quantitative RT–PCR were enhanced by MPs^{Shh+} treatment and this effect was abolished by cyclopamine (Figure 4C). The increased ICAM-1 mRNA expression upon MPs^{Shh+} treatment was associated with enhanced protein expression (Figure 4D). In order to confirm the ability of MPs^{Shh+} to favor cell adhesion, we assessed the effects of MPs^{Shh+}-induced adhesion on Eahy cells using fibrinogen-coated plates. Under these conditions, adhesion was enhanced by MPs^{Shh+} treatment, suggesting an interaction between fibrinogen and its receptor, ICAM-1 (Figure 4A).

Then, the effect of Y-27632 on the expression and activation of proteins linked to cell adhesion was investigated. MPs^{Shh+} induced an increase in Rho A expression and FAK phosphorylation as evidenced by immunolabeling and western blot. These effects were inhibited not only by Y-27632 but also by cyclopamine (Figure 5A–C). In addition, labeling of actin fibers with phalloidin showed that MPs^{Shh+} induced the formation of stress fibers, which was reversed by Y-27632 treatment (data not shown). These results suggest that proteins such as Rho A and FAK, which are involved in cell adhesion, are upregulated by MPs^{Shh+} through both ROCK and Shh pathways, despite the existence of pathways not associated with Smo receptor activation.

MPs^{Shh+} upregulate proangiogenic factors through the activation of the Shh pathway

Angiogenic factors were analyzed by real-time quantitative RT–PCR using a panel of different human angiogenic factor mRNA. Among the 46 transcripts studied (Table I), those for proangiogenic factors such as hepatocyte growth factor, metalloproteinase-1, IL-1β and VEGF A and its receptor FLT-1 were increased by MPs^{Shh+} treatment. These effects were partially inhibited by cyclopamine. In contrast, transforming growth factor (TGF)-β2 mRNA was reduced by MPs^{Shh+}, independently of the inhibition of the Shh pathway (Figure 6A).

In order to confirm that MPs^{Shh+} treatment upregulated proangiogenic factors, protein levels of VEGF, FLT-1 and IL-1β were analyzed. Western blot analyses show that neither IL-1β nor FLT-1 protein levels were increased when cells were incubated in the presence of MPs^{Shh+}. In contrast, VEGF protein expression was increased by MPs^{Shh+} and these effects were reversed by cyclopamine (Figure 6B).

Discussion

In the present study, we demonstrate that MPs generated from activated/apoptotic T cells harboring Shh differentially regulate the steps implicated in angiogenesis. Thus, MPs^{Shh+} increased capillary-like formation through the increase of cell adhesion, the upregulation of proteins such as ICAM-1 and Rho A and the activation of FAK and proangiogenic factors, mainly VEGF, via the activation of the Shh pathway. In addition, the effects induced by MPs^{Shh+} on cell adhesion were dependent on ROCK pathway. However, MPs^{Shh+} reduced cell
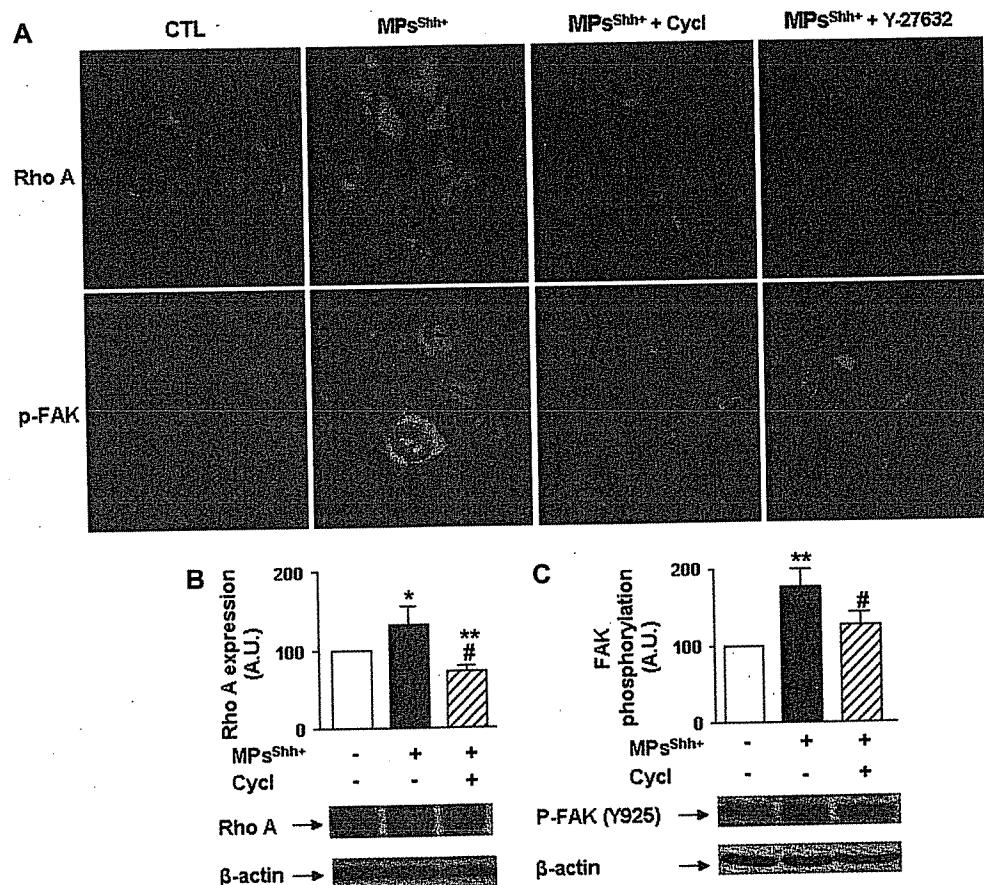
Fig. 5. MPs^{Shh+} increase expression of Rho A and phosphorylation of FAK. (A) Immunofluorescence staining of endothelial cells for Rho A and p-FAK showed upregulation of Rho A expression and activation of FAK pathway after MPs^{Shh+} treatment. These effects were reversed by either cyclopamine (Cycl, 30 µM) or Y-27632 (10 µM) treatment. (B and C) This was confirmed by western blot. β-Actin control is included. Data are representative of five separate blots, and the densitometry values are expressed in arbitrary units (A.U.) as mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus in the absence of MPs^{Shh+}. #$P < 0.05$ versus in the presence of MPs^{Shh+} alone.

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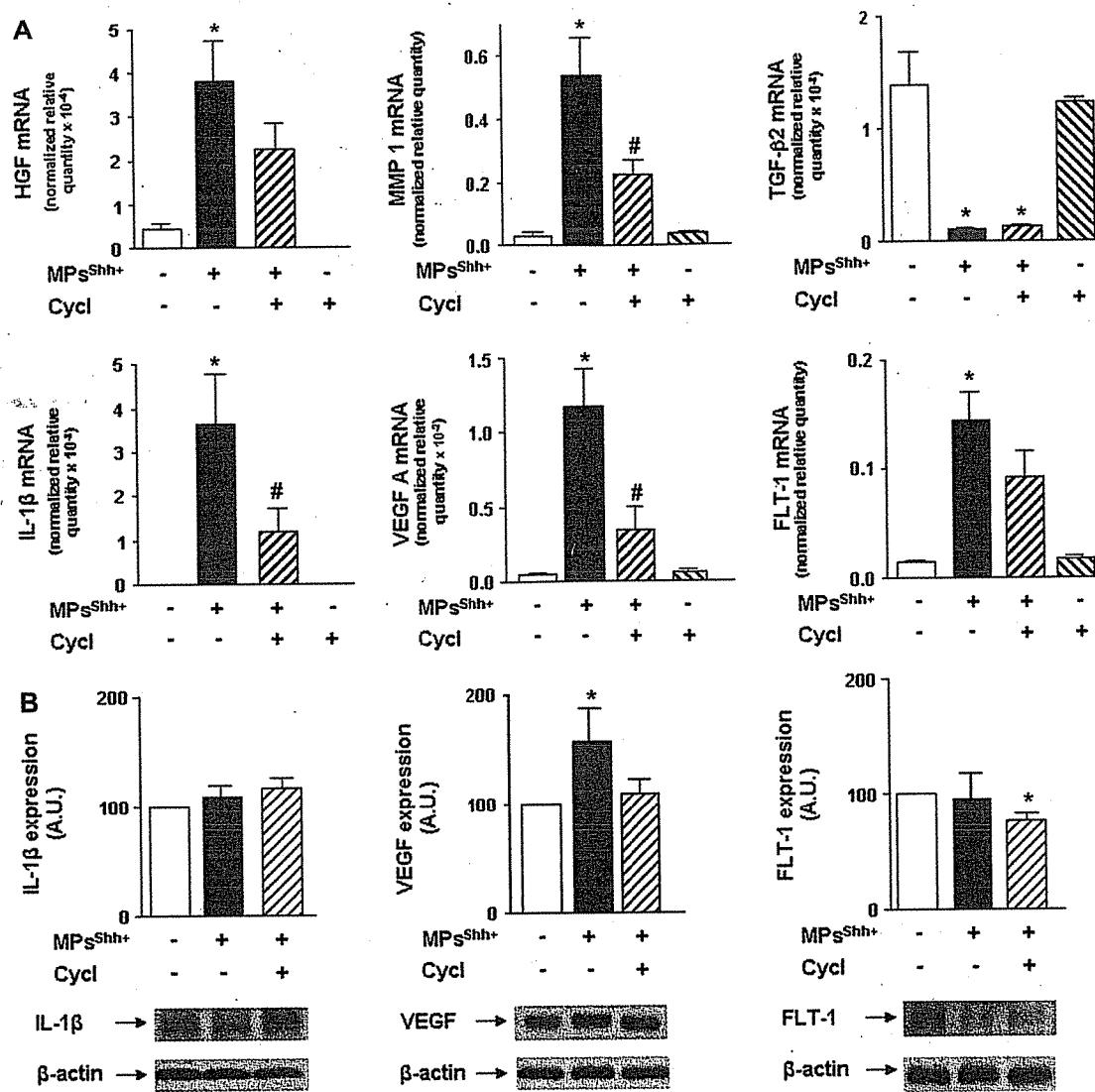
Fig. 6. MPs^{Shh+} modify mRNA and protein expressions of angiogenic factors. (A) Quantitative RT–PCR analysis was conducted on total RNA from six independent Eahy cultures. HGF, MMP 1, VEGF A, FLT1 and IL-1β mRNA expression levels were enhanced by MPs^{Shh+} treatment. Cyclopamine partially reversed the MPs^{Shh+}-induced increase of VEGF A, IL-1β and MMP 1 mRNA expressions. TGF-β2 mRNA expression was reduced by MPs^{Shh+} treatment. *$P < 0.05$ versus in the absence of MPs^{Shh+}. #$P < 0.05$ versus in the presence of MPs^{Shh+} alone. (B) Western blot showing the protein expression of IL-1β, VEGF A and FLT-1. *$P < 0.05$ versus in the absence of MPs^{Shh+}.

migration and proliferation through the mechanisms independent of Smo receptor activation. These data suggest that MPs transfer a biological message carried by Shh that can account for a large number of events associated with angiogenesis.

In tumor-associated angiogenesis, angiogenic factors secreted by endothelial and tumor cells stimulate endothelial cells to degrade the vascular basal membrane and migrate into surrounding tissues promoting the proliferation of solid tumors (31). Here, we showed that MPs^{Shh+} are able to increase expression level of mRNA of proangiogenic factors such as VEGF A, FLT-1, ICAM-1, metalloproteinase-1, IL-1β and hepatocyte growth factor, and these effects were Smo dependent. Among these factors, the complex VEGF A–FLT-1 represents the major player in angiogenesis initiation by inducing endothelial nitric oxide production (32), in parallel to the increase of ICAM-1 expression (33). Surprisingly, while protein level of VEGF was increased by MPs^{Shh+} treatment, its receptor, FLT-1, did not. Several hypotheses can explain these results. On one hand, it is plausible that expression of VEGF receptor is delayed when compared with its mRNA expression. On the other hand, it is possible that the increase of translation to FLT-1 protein is performed at low levels that may be below the threshold of detection for the antibody

used in the present study, as described previously in astrocytes by Krum *et al.* (34). Nevertheless, the proangiogenic factor VEGF is overexpressed suggesting that MPs^{Shh+} could induce angiogenesis through a VEGF-dependent mechanism. However, the conditioned media obtained from cells treated with MPs^{Shh+} was not able to favor formation of capillaries. These results indicate that, probably, the amount of proangiogenic factors produced by MPs^{Shh+} may not be sufficient to induce angiogenesis and alternatively a direct stimulation of endothelial cells by Shh associated with MPs is mandatory to favor formation of capillary-like structures. Also, MMPs are essential for angiogenesis due to their ability to degrade the components of the extracellular matrix and thus MMPs participate in the remodeling of basement membranes (35). Moreover, IL-1β induces VEGF A expression through the phosphatidylinositol 3-kinase pathway (36). Although protein levels of IL-1β are not increased, as for VEGF receptor, IL-1β protein expression might be delayed when compared with its mRNA expression. Finally, hepatocyte growth factor is identified as a member of angiogenic growth factors with a potent action on human endothelial cells (37). Interestingly, expression of TGF-β2 was downregulated by MPs^{Shh+}. TGF-β acts as a tumor suppressor early in carcinogenesis, but then switches to

a tumor promoter later by affecting both the cancer cells and the tumor microenvironment (38). Both TGF-β2 and Shh are involved in processes during embryonic development and cancer. Indeed, it has been recently shown that TGF-β induces the expression of the Hedgehog-signaling molecules Gli1·and Gli2 in various cancer cell lines independently of the Hedgehog receptor activation (39). The fact that MPs^{Shh+} reduced TGF-β2 expression may favor angiogenesis. Regarding the effects of Shh on angiogenic factors, it has been reported that recombinant Shh is able to induce the expression of angiopoietin-1 and reduce angiopoietin-2 expression in embryonic fibroblast cells (40). In contrast, angiopoietin-1, angiopoietin-2 and VEGF are overexpressed by recombinant Shh treatment in human fibroblasts (5), whereas Shh-deficient mice present a downregulation of mRNA expression of angiopoietin-1 but not of angiopoietin-2 and VEGF (41). Altogether, the present study shows that Shh carried by MPs acts on a large number of target genes that regulate angiogenesis at different phases.

In the present study, MPs^{Shh+} upregulated Rho A expression, as shown by western blot and confocal microscopy, and induced the phosphorylation of FAK that were reversed by both ROCK inhibitor and cyclopamine, suggesting a cross-link between ROCK and Shh pathways. Also, formation of stress fibers by MPs^{Shh+} was dependent of ROCK pathway as demonstrated by the lack of effects of MPs^{Shh+} when the ROCK inhibitor was present (data not shown). It has been shown that the activities of Rho A and ROCK are responsible for several effects of Shh activation (42,43). Paradoxically, MPs^{Shh+} increased endothelial cell adhesion by a mechanism sensitive to ROCK inhibitor but not cyclopamine. The lack of effect of cyclopamine in cell adhesion might be due to the complexity of this process or the implication of a·mechanism independent to Smo receptor activation. Thus, Shh pathway may be involved in the activation of certain (here, Rho A and p-FAK), but not all, proteins implicated in the adhesion process. Nevertheless, MPs^{Shh+} enhanced endothelial cell adhesion indicating a role of these MPs in angiogenesis.

Both cell migration and proliferation were inhibited by MPs^{Shh+} and these effects were independent of Smo receptor activation, suggesting that although formation of capillary-like structures is promoted by MPs^{Shh+}, these MPs are able to differentially regulate cell events leading to angiogenesis. Similar results have been reported for the angiopoietin-like 4, which is able to promote angiogenesis (44) and inhibit endothelial cell migration by an interaction with the extracellular matrix (45). Moreover, it should be noted that the nature of all molecular components of MPs^{Shh+} is not known. Proteomic studies have shown that MPs composition is complex and dependent on the stimuli used for their generation as well as on the cell origin (46,47). In the present study, we have engineered MPs harboring Shh but other components are not determined and, obviously, they can influence angiogenesis.

In summary, MPs carrying Shh regulate multiple pathways related to *in vitro* angiogenesis, mainly through the production of proangiogenic factors and upregulation of proteins involved in cell adhesion. Expression of Shh correlates with the tumorigenesis of different types of cancer such as basal cell carcinoma, pancreatic and prostatic cancer and gliomas (48–51). In addition, release of MPs from tumor or vascular cells is also related with tumorigenesis. Thus, targeting Shh pathway would represent a novel therapeutic tool that can regulate angiogenesis and in consequence, tumor development.

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